

(19)



Europäisches Patentamt  
European Patent Office  
Office européen des brevets

(11) Publication number:

**0 318 775**  
**A2**

(12)

# EUROPEAN PATENT APPLICATION

(21) Application number: 88119211.6

(51) Int. Cl.4: **C12N 15/00 , C07H 21/04 ,**  
**C12N 1/20 , C12N 9/18 ,**  
**/(C12N1/20,C12R1:19)**

(22) Date of filing: 18.11.88

A request for correction of the originally filed description has been filed pursuant to Rule 88 EPC. A decision on the request will be taken during the proceedings before the Examining Division (Guidelines for Examination in the EPO, A-V, 2.2).

(30) Priority: **03.12.87 JP 306638/87**  
**20.05.88 JP 123672/88**  
**27.07.88 JP 187684/88**

(43) Date of publication of application:  
**07.06.89 Bulletin 89/23**

(64) Designated Contracting States:  
**BE CH DE FR GB LI**

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(54) **A lipase gene.**

(57) A process for improving conventional lipase-modification having an inferior efficiency is provided, which process comprises cloning the structural gene of a lipase present in the chromosome of Pseudomonas genus bacterium, determining a DNA arrangement coding the lipase, obtaining a recombinant DNA containing the DNA arrangement and using Escherichia coli containing the recombinant DNA to obtain a lipase originated from Pseudomonas genus bacterium or its variant, utilizable for commercial use applications, the nucleotide of the DNA arrangement coding the lipase having the following arrangement:

**EP 0 318 775 A2**

10	20	30	40	50	60
ATGGACGATT	CGGTAAATAC	ACGCTATCCG	ATCTTATTGG	TACACGCGCT	TTTTGGATTG
70	80	90	100	110	120
GACCGGATAA	GCTCGCATEA	CTACTTTTCAT	GGTATCAAAG	AAGCACTCAA	TGAGTGGCGT
130	140	150	160	170	180
GCCAGCOTCT	TCGTTCCAAT	CATTTTCAGC	GCCAACGACA	ATGAAGCCCG	AGGCGATCAA
190	200	210	220	230	240
CTGCTCAAAG	AGATCCACAA	CCTGCGCAGG	CAGGTCGGTG	CACAGCOTGT	CAACCTGATC
250	260	270	280	290	300
GGCCACAACC	AGGCGCGCCCT	TACCGCGCGT	TATGTGGCGG	CCATCGCCCC	TGAACCTGATC
310	320	330	340	350	360
GCCTCGGTGA	CGTCAGTCA	TGCCCCCAAC	CACGGCTCCG	AGCTGGCCGA	TGGCTTGCGC
370	380	390	400	410	420
CTGGCCTTTG	TCCCGGGGAG	GCTTGCGCAA	ACGGTGGCTG	CCCGCCTGAC	CACCTCGTTC
430	440	450	460	470	480
AGCGCATTTT	TATCGCGCCCT	CAGCGGCCAC	CCCGCGCTGC	CCCAAAATGC	CTTGAATGCG
490	500	510	520	530	540
CTTAACGCCC	TGACCACCGA	TGGGCTTGCC	GCCTTCAACC	GCCAATATCC	CCAGGGGCTG
550	560	570	580	590	600
CCCGACAAGT	GGGCGCGCAT	GGGCCCCGCA	CAGGTGAATG	CTGTGCACTA	TTATTCCTGG
610	620	630	640	650	660
AGCGGCATCA	TCAAAGGCTC	GCGGCTGGCC	GAGTCACTCA	ACCTGCTCGA	CCCCTTGCAC
670	680	690	700	710	720
AATGCCTTGC	GTGTGTTTGA	CACTTTTTTT	ACCGCGGAAA	CCCGAGAAAA	CGACGGCATG
730	740	750	760	770	780
GTTGGCCGCT	TCAAGTCGCA	TCTGGGCGAG	GTCATCGGCT	CCGACTACCG	TCTGGACCAC
790	800	810	820	830	840
CTCGACACCA	TCAACCATAT	GGCAAGAGGA	AGCGCAGGCG	CATCAACCCG	GTAG

## A lipase gene

## BACKGROUND OF THE INVENTION

## 5 1. Field of the Invention

This invention relates to a DNA arrangement coding a lipase originated from Pseudomonas genus bacterium, a recombinant DNA containing the DNA arrangement and Escherichia coli containing the same and further a process for producing the lipase.

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## 2. Description of the Related Art

Lipases have been used as a lipid-hydrolyzing enzyme, for oils- and fats-processing, medicine for  
15 clinical diagnosis, detergents, digestants, etc. Further, in recent years, they are important enzymes which catalyze ester hydrolysis, ester synthesis or ester conversion in the production of chemical products, particularly optically active compounds.

Further, Pseudomonas genus bacterium has been known to produce lipases useful as a catalyst for ester hydrolysis, ester synthesis or ester conversion (Japanese patent application laid-open No. Sho 62-  
20 166,898/1987).

The specific feature of the lipases produced by the Pseudomonas genus bacterium is interesting in the aspect of commercial utilization, but in order to achieve the object, a more substrate-specificity (including steric specificity) has been desired.

However, since the primary structure and higher-order structures thereof are unknown, unless the  
25 improvement is relied upon a process of inferior efficiency such as conduction of an enormous screening, conduction of a number of times of mutation treatments, etc., it has been impossible to obtain lipases having a preferred activity.

Further, since lipases are produced by expression of usually one structural gene contained in the chromosome DNA of the production bacteria, it has been difficult to improve the production of lipases with a  
30 leap.

In recent years, due to development of recombinant DNA technique, it has become possible to derive genes into those having a higher activity through its isolation or to transform nucleotide arrangement; thus it has been investigated or conducted to originate commercially interested proteins.

The present inventors have made extensive research in order to improve the above-mentioned  
35 conventional processes for modifying lipases, which processes, however, have been inferior in the efficiency.

## SUMMARY OF THE INVENTION

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The object of the present invention is to clone the structural gene of a lipase present in the chromosome of Pseudomonas genus bacterium to thereby determine a DNA arrangement coding the lipase, and obtain a recombinant DNA containing such a DNA arrangement and further by using Escherichia coli containing such a recombinant DNA, obtain a lipase originated from Pseudomonas genus bacterium or  
45 its variant retaining substantially the same specific features as those of the above-mentioned lipase, which lipase and variant are utilizable for commercial use applications.

As a result, the present inventors have succeeded in cloning of the structural gene of a lipase present in the chromosome of Pseudomonas fragi IFO 12049 strain, determination of a DNA arrangement coding the lipase and production of the lipase by the medium of Escherichia coli, and have achieved the present  
50 invention.

The present invention has the following constitutions (1) to (8):

(1) A DNA arrangement coding a lipase originated from Pseudomonas genus bacterium the nucleotide of which lipase has the following arrangement or a DNA arrangement coding a functional equivalent to said nucleotide arrangement:

```

      10      20      30      40      50      60
ATGGAACGATT CGGTAAATAC ACCTATATCCG ATCTTATTGG TACACGGCCT TTTTGGATTG

      70      80      90     100     110     120
5  GACCGGATAG GCTCGCATCA CTACTTTTCAT GOTATCAAGC AAGCACTCAA TGAGTGCGGT

      130     140     150     160     170     180
      GCCAGCOTCT TCGTTCCAAT CATTTCAGCC GCCAACGACA ATGAAGCCCG AGCGCATCAA

10  CTGCTCAAAGC AGATCCACAA CCTGCGCAGG CAGGTGCGGT CACAGCGTOT CAACCTGATC

      250     260     270     280     290     300
15  GGCCACAAGC AGGGCGCCCT TACCGCGCGT TATGTGGCGG CCATCGCCCC TGAAGTGATC

      310     320     330     340     350     360
      GCCTCGGTGA COTCAGTCAQ TGCCCCCAAC CACGGCTCCG AGCTGGCCGA TCGCTTGCGC

20  CTGGCCTTTG TCCCAGGAGG GCTTGGCGAA ACGGTGGCTG CCGCCCTGAC CACCTCGTTC

      430     440     450     460     470     480
      AGCGCATTTT TATCGGCCCT CAGCGGCCAC CGCGGCCTGC CCCAAAATGC CTTGAATGCG

25  CTTAACGCCC TGACCACCGA TGGGOTTGCC GCCTTCAACC GCCAATATCC CCAGGGGCTG

      550     560     570     580     590     600
30  CCGGACAGAT GGGGCGGCAT GGGCCCCGCA CAGGTGAATG CTGTGACTA TTATTCCTGG

      610     620     630     640     650     660
      AGCGGCATCA TCAAAGGCTC GCGGCTGGCC GAGTCACTCA ACCTGCTCGA CCCCTTGAC

35  AATGCCTTGC GTGTGTTTGA CAGCTTTTTT ACCCGCGAAA CCCGAGAAAA CGACGGCATG

      730     740     750     760     770     780
      GTTGGCCGCT TCAGCTCGCA TCTGGGCCAG GTCATCCGCT CCGACTACCC TCTGGACCAC

40  CTCGACACCA TCAACCATAT GGCAAGAGGA AGCGCAGGCG CATCAACCCG GTAG

      790     800     810     820     830     840

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(2) a DNA arrangement according to item (1) wherein said *Pseudomonas* genus bacterium is *Pseudomonas fragi* IFO 12049 strain;

(3) a recombinant DNA replicable inside *Escherichia coli*, having a DNA arrangement coding said lipase originated from *Pseudomonas* genus bacterium or a DNA arrangement coding a functional equivalent to said nucleotide arrangement, each confirmed in item (1), incorporated into a vector for *Escherichia coli*;

(4) a recombinant DNA according to item (3), wherein said *Pseudomonas* genus bacterium is *Pseudomonas fragi* IFO 12049 strain;

(5) a recombinant DNA according to item (3), wherein said vector for *Escherichia coli* is pUC9;

(6) a recombinant DNA according to item (3), wherein said *Pseudomonas* genus bacterium is *Pseudomonas fragi* IFO 12049 strain and said vector for *Escherichia coli* is pUC9;

(7) an *Escherichia coli* transformed by a recombinant DNA according to item (3);

(8) an *Escherichia coli* transformed by a recombinant DNA according to item (3) wherein said *Pseudomonas* genus bacterium is *Pseudomonas fragi* IFO 12049;

(9) an *Escherichia coli* transformed by a recombinant DNA according to item (3) wherein said vector for *Escherichia coli* is pUC9;

(10) a process for producing a lipase which comprises using a recombinant DNA according to item (3);

5 (11) a process for producing a lipase which comprises cultivating an *Escherichia coli* according to item (7);

(12) a process for producing a lipase which comprises cultivating an *Escherichia coli* according to item (7) wherein said *Pseudomonas* genus bacterium is *Pseudomonas fragi* IFO 12049; and

10 (13) a process for producing a lipase which comprises cultivating an *Escherichia coli* according to item (7) wherein said vector for *Escherichia coli* is pUC9.

#### BRIEF DESCRIPTION OF THE DRAWINGS

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Figs. 1 to 5 each show an explanatory chart for illustrating the present invention.

Fig. 1 shows the DNA arrangement of a lipase structural gene originated from *Pseudomonas fragi* IFO 12049 strain.


20 Fig. 2 shows the restriction enzyme map of plasmid pFL-1. The portion of  refers to a DNA fragment originated from *Pseudomonas fragi* IFO 12049 strain chromosome DNA.

Fig. 3 shows the respective inserted fragments of plasmids pFL-2, pFL-2SC, pFL-2ND, pFL-2AB and pFL-2SM into a plasmid pUC9 and the presence or absence of formation of clear zones in a tributyrin agar medium.

Clear zone "+" indicates formation of clear zone.

25 Fig. 4 shows a chromosome DNA arrangement (a linker being partly contained in a plasmid pUC9) containing a lipase structural gene of *Pseudomonas fragi* IFO 12049 strain of a plasmid pFL-2 and an amino acid arrangement corresponding to the lipase DNA arrangement.

Fig. 5 shows *Escherichia coli* JM83 (pUC9) strain (lane a) and JM83 (pFL-2) strain (lane b) analyzed with a polypeptide according to 15% SDS-polyacrylamide gel electrophoresis.

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The numeral figures on the left side each show the molecular weight ( $\times 1,000$ ) of protein marker simultaneously subjected to electrophoresis.

#### 35 DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The recombinant DNA of the present invention is obtained by fragmenting the chromosome DNA of *Pseudomonas* genus bacterium producing a lipase, incorporating the resulting fragment into a multicopy type plasmid vector replicating the fragment inside *Escherichia coli* and selecting, from among the resulting

40 recombinant DNAs, that containing a lipase structural gene.

The chromosome DNA of the *Pseudomonas* genus bacterium is prepared by subjecting the bacterial body *Pseudomonas* genus bacterium treated with lysozyme to lytic reaction with a proteolytic enzyme in the presence of sodium dodecylsulfate (SDS), followed by extracting the resulting lytic substance with phenol to carry out protein-removal and precipitating the DNA with ethanol.

45 The fragmentation of the chromosome DNA is carried out with an endonuclease having no base arrangement specificity.

Using *Sau* 3A1 for fragmenting the *Pseudomonas* chromosome DNA and using pUC9 (J. Viera and J. Messing, *Gene*, 19, 259 (1982)) as *Escherichia coli* plasmid vector, it is possible to introduce the chromosome DNA fragment into the *Bam* H1 site as a cloning site.

50 Here, pUC9 is used as DNA-introducing vector for *Escherichia coli*, but in the present invention, any kind may be used as far as it can be replicated inside *Escherichia coli*, and has a suitable selective marker and the character expression of a foreign gene can be expected therefrom. For example, pUC8, pUC18, pUC19, etc. may be used.

55 The chromosome DNA fragment of the *Pseudomonas* genus bacterium decomposed with a restriction enzyme is separated according to electrophoresis in agarose gel and visualized by dyeing with ethidium bromide under ultraviolet rays and the resulting DNA fragment having a desired size is adsorbed onto a DEAE cellulose paper. The adsorbed substance is dissolved away with 1M NaCl solution and recovered by precipitation with ethanol.

Introduction of the chromosome DNA of the *Pseudomonas* genus bacterium into the plasmid vector is achieved by cleaving the chromosome DNA and the plasmid vector by means of a restriction enzyme and link both the resulting fragments together.

In advance of the linking, the cleaved vector is dephosphorylated by a bacterial alkaline phosphatase to prevent self-linking. The chromosome DNA fragment and the plasmid vector can be linked with a ligase.

*Escherichia coli* is transformed with the combinant DNA obtained through the above-mentioned steps and *Escherichia coli* having a lipase productivity is selected based on the marker of the plasmid vector and a lipase productivity.

As to *Escherichia coli*, as those which have an adequate marker afforded by inserting a specified cloning vector such as ampicillin-resistant bacterium selected, JM83 strain (M. Messing, R. Crea and P.H. Seeburg, Nucl. Acid. Res., 2309 (1981) may be used. Further, it is also possible to use *Escherichia coli* such as HB101, LE392, etc.

The transformation is carried out according to well-known competent cell method, and the selection by means of the plasmid marker is carried out by adding ampicillin (about 50  $\mu\text{g/ml}$ ) to a medium.

Further, the choice through the productivity of the lipase may be carried out by adding tributyrin (about 1%) to the medium. Namely, such a phenomenon is utilized that when the lipase is formed on the medium, a lipid is decomposed into fatty acids and the emulsified state of the lipid changes to form a circular clear zone on the periphery of the resulting colony (W. Kugiyama, Y. Otani, Y. Hashimoto and Y. Takagi, Biochem. Biophys. Res. Commun., 141, 185 (1986)).

The recombinant DNA containing the lipase structural gene obtained through the above-mentioned steps is decomposed by various restriction enzymes; a map of the restriction enzymes is prepared based on measurement according to electrophoresis method using agarose gel; and subcloning of the present recombinant DNA is carried out.

Namely, when the resulting recombinant DNA is broken with a restriction enzyme; DNA fragments having a suitable size (larger than that of expected lipase structural gene, but not excessively larger than that) are collected; and these fragments are again incorporated into the plasmid vector for *Escherichia coli*, or the recombinant DNA is broken with a restriction enzyme; an unnecessary DNA portion is removed; and the resulting material is reunited as it is, then it is possible to reduce the molecular weight of the recombinant DNA.

Fig. 2 shows an example of the restriction enzyme map of plasmid pFL-1 containing the lipase structural gene of *Pseudomonas fragi* IFO 12049 strain.

Fig. 3 shows an example of the presence or absence of formation of clear zone in a tributyrin agar medium, of *Escherichia coli* having the respective plasmids pFL-2, pFL-2SC, pFL-2ND, pFL-2AB and pFL-2SM, having DNA fragments broken by means of various restriction enzymes, Eco RI, Sca I, Nde I, Apa I and Sma I of plasmid pFL-1, inserted therein.

Determination of the DNA region wherein the DNA arrangement coding the lipase originated from *Pseudomonas* genus bacterium is present can be made according to a known method such as Sanger et al's dideoxy method (Sanger, F. et al, Proc. Natl. Acad. Sci., USA 74, 5463, 1977).

As to whether or not the DNA arrangement recited in Fig. 1 relative to the above-mentioned item (1), initiated from an initiation codon ATG and terminated by a termination codon TAG, actually corresponds to the arrangement of the lipase produced by means of *Escherichia coli* transformed by plasmid pFL-2, the correspondence has been judged from a fact that expression of a polypeptide having a molecular weight of ca.33,000 determined according to the above-mentioned defect experiment and SDS-polyacrylamide gel electrophoresis has been confirmed.

Namely, the DNA arrangement of the lipase structural gene of *Pseudomonas* IFO 12049 strain contained in the plasmid pFL-2, has been judged from a fact that the above arrangement includes an arrangement recognized by the restriction enzyme Nde I but does not include an arrangement recognized by Sca I and it is of an open reading frame wherein the molecular weight is ca. 33,000.

Fig. 4 shows a chart of a chromosome DNA arrangement (partly containing the linker of the plasmid pUC9) including a region wherein the lipase of the *Pseudomonas fragi* IFO 12049 strain of the plasmid pFL-2 is coded and an amino acid arrangement corresponding to the DNA arrangement of the lipase gene.

When *Escherichia coli* is transformed with a recombinant DNA containing a lipase structural gene originated from *Pseudomonas* genus bacterium, which recombinant DNA has been reduced in the molecular weight at the above-mentioned subcloning step, then it is possible to again obtain *Escherichia coli* capable of producing the lipase.

The constituent of the *Escherichia coli* capable of producing the lipase is separated up to a polypeptide level according to electrophoresis in SDS-polyacrylamide gel. The thus separated polypeptide is dyed in Coomassie Brilliant Blue and visualized.

Expression in *Escherichia coli*, of the lipase structural gene originated from *Pseudomonas* genus bacterium, which gene has been incorporated into a vector for introducing DNA for *Escherichia coli*, is confirmed by cultivating the above *Escherichia coli* and an *Escherichia coli* having no heterogeneous structural gene incorporated therein and containing only the above-mentioned vector for introducing DNA for *Escherichia coli*, respectively; separating the thus prepared samples into polypeptides; visualizing them; and then comparing the resulting materials.

For example, Fig. 5 shows a view of polypeptides separated in SDS-polyacrylamide gel and visualized, with *Escherichia coli* JM83 (pUC9) strain ("a" in Fig. 5) and *Escherichia coli* JM83 (pFL-2) strain ("b" in Fig. 5).

By comparing the above two samples, expression in *Escherichia coli*, of the lipase structural gene of *Pseudomonas fragi* IFO 12049 strain originated from plasmid pFL-2 is confirmed.

Further, in order to ensure as high an expression level as possible, of the heterogeneous structural gene incorporated into the *Escherichia coli*, it is necessary to relate the above-mentioned gene to as effective a promotor-operator system as possible.

In the case of *Escherichia coli*, various effective promotor-operator systems have been known such as lactose (lac) operon, tryptophan (trp) operon, outer membrane protein gene (lpp) promotor,  $\beta$ -lactamase promotor, etc.

These various methods may be employed for expression in *Escherichia coli* within a region wherein the lipase of the present invention is coded. Further, in order to obtain similar results, various replication-initiating sites, marker genes, effective promoters and other structural elements may be combined.

Transformed *Escherichia coli*s obtained in the above-mentioned various cases, too, are within the scope of the present invention.

Further, the reproduction conditions may be determined depending on the specific features of *Escherichia coli* strains so that the lipase can be reproduced most efficiently.

The present invention will be described in more detail by way of Examples, but it should not be construed to be limited thereto.

All of the restriction enzymes used in Examples are commercially available products made by NIPPON GENE CO., LTD.

### Example 1

(Preparation of *Pseudomonas fragi* IFO 12049 strain chromosome DNA)

*Pseudomonas fragi* IFO 12049 strain was cultivated in L-medium (containing Bactotryptone (1%), a yeast powder (0.5%) and NaCl (0.5%) and adjusted to pH 7.2) (200 ml) at 30°C overnight, followed by collecting the resulting bacterial bodies, once washing the bacterial bodies with 10 mM-Tris-HCl buffer solution (pH 8.0) and 30-mM NaCl solution (10 ml) and suspending them in 10 mM-Tris-HCl buffer solution (pH 8.0) and 1mM-EDTA 2Na solution (12 ml), based on 100 ml of the culture solution.

To the resulting suspension were added Tris-HCl buffer solution, Tris 2Na, EDTA-HCl buffer solution (pH 8.0) and lysozyme (made by Seikagaku Kogyo Company) so that their final concentrations could be 50 mM, 50 mM, 50 mM and 1 mg/ml, respectively, followed by allowing the resulting solution to stand at room temperature (25°C) for one hour.

To the solution was added Proteinase K (trademark of product made by Merck Company) in the presence of 0.5% SDS so as to give a concentration of 100  $\mu$ g/ml, followed by slowly shaking the mixture at 50°C for 3 hours to dissolve the bacterial bodies.

The resulting sample was twice extracted with an equal quantity of Tris-saturated phenol, followed by precipitating the extract with ethanol, dissolving the precipitates in 10 mM Tris-HCl buffer solution (pH 8.0) -1mM-EDTA solution (hereinafter abbreviated to TE solution), and subjecting the solution to dialysis at 4°C for 2 days to prepare a chromosome DNA.

### Example 2

(Cloning of lipase gene)

*Pseudomonas fragi* IFO 12049 strain chromosome DNA (about 7  $\mu$ g) prepared in Example 1 was partially digested with 1U *Sau* 3AI at 37°C according to reactions with lapse of times of 5, 15, 25, 35 and 50 minutes, followed by stopping the reactions with EDTA 2Na solution so as to give a final concentration of 20 mM and mixing the respective reaction solutions.

The resulting sample obtained by the mixing was subjected to electrophoresis with 40 mA for 16 hours in 0.7% agarose gel in 40 mM Tris, 2 mM sodium acetate and 1 mM EDTA 2Na, at pH 7-9.

DNA fragments of 2-6 Kb in the agarose gel were adsorbed onto Whatman DE81 DEAE cellulose paper (tradename of product made by Whatman Company), followed by dissolving out the adsorbed substance with 300  $\mu$ l 1M-NaCl-TE solution, twice extracting the resulting dissolved-out substance with an equal quantity of 300  $\mu$ l 1M-NaCl-TE solution and adding ethanol in 2.5 times the quantity of the extract to precipitate and recover it.

The quantity of the substance recovered at that time was 0.7  $\mu$ g. This sample and pUC9 (1.5  $\mu$ g) obtained by digesting *Dam* HI, followed by dephosphorizing the resulting substance with 0.6 U alkaliphosphatase (made by Takara Shuzo Company) at 50°C for 3 hours were linked by means of T4 DNA ligase of 1  $\mu$ l (147 U/ $\mu$ l) in the presence of Tris-HCl buffer solution (pH 8.0) (66 mM),  $MgCl_2$  (6.6 mM), ATP (0.066 mM) and dithiothreitol (10 mM) at 4°C for 16 hours.

Using the thus linked DNA sample, JM 83 strain of  $\sim 2 \times 10^9$  bacterial bodies was transformed according to competent cell method, followed by cultivating the resulting substance for 16 hours on L-agar medium containing Ampicillin (trademark of product made by Meiji Seika Company) (50  $\mu$ g/ml), tributyrin (1%) and X-gel (0.0002%).

Among about 1500 transformed strains selected by marker of pUG9 plasmid vector, there was one strain which formed a clear zone in the medium. Thus this strain was separated and named *Escherichia coli* 83 (pFL-1).

### Example 3

(Analysis of recombinant DNA)

Plasmid pFL-1 was digested with various restriction enzymes, followed by preparing a restriction enzyme map based on measurement according to agarose gel electrophoresis. The restriction enzyme map of pFL-1 is shown in Fig. 2.

Based on the restriction enzyme map of plasmid pFL-1, the respective DNA fragments obtained by breakage with the various restriction enzymes were subcloned into pUC9 to transform *Escherichia coli* JM 83 strain, followed by observing the presence or absence of formation of clear zone in a medium containing tributyrin (1%) as in the case of Example 2.

Fig. 3 shows the presence or absence of formation of clear zone of *Escherichia coli* having plasmids i.e. pFL-2, pFL-2SC, pFL-2ND, pFL-2AB and pFL-2SM, having DNA fragments obtained by breaking plasmid pFL-1 with various restriction enzymes i.e. *Eco* RI, *Sca* I, *Nde* I, *Apa* I or *Sma* I, inserted into the respective plasmids.

### Example 4

(Determination of DNA arrangement)

As to the portion originated from the chromosome DNA of *Pseudomonas fragi* IFO 12049 strain of plasmid pFL-2, DNA arrangement was determined with M13 Sequencing Kit (trademark of product made by Takara Shuzo Company) and Deaza Sequencing Kit (trademark of product made by NIPPON GENE CO., LTD.). As a result, an open reading frame shown in Fig. 4 was confirmed.

### Example 5

(Expression of recombinant DNA in *Escherichia coli*)



Escherichia coli JM 83 (pUC9) strain and JM 83 (pFL-2) strain were cultivated in a shaking culture machine at 37° for 16 hours, in a L liquid medium containing 50 µg/ml of Ampicillin (trademark of product made by Meiji Seika Company). The resulting bacterial bodies were collected, followed by again suspending the bodies in 0.1 M phosphoric acid buffer solution (pH 0.7), mixing the bacterial bodies corresponding to the culture solution (30 µl) with an equal quantity of 2X sample buffer solution (10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS and 0.0625 M Tris-HCl (pH 6.8), boiling the mixture for 5 minutes and analyzing with polypeptide according to 15% SDS-polyacrylamide gel electrophoresis.

The SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli et al's method (Laemmli, U.K. Nature 227 680 (1970)), and the electrophoresis was carried out with 20 mA till Bromophenol Blue reached the lower end of the gel.

Fig. 5 shows polypeptides relative to Escherichia coli JM 83 (pUC9) strain (lane a) and JM 83 (pFL-2) strain (lane b) dyed by Coomassie Brilliant Blue solution in 15% SDS-polyacrylamide gel. Expression (polypeptide shown by an arrow mark (←)) in Escherichia coli JM 83 strain of a lipase structural gene originated from Pseudomonas fragi IFO 12049 strain contained in plasmid pFL-2 was confirmed.

### Claims

1. A DNA arrangement coding a lipase originated from Pseudomonas genus bacterium the nucleotide of which lipase has the following arrangement or a DNA arrangement coding a functional equivalent to said nucleotide arrangement:

```

      10      20      30      40      50      60
ATGGACGATT CGGTAAATAC ACGCTATCCG ATCTTATTGG TACACGGCCT TTTTGGATTC

5      70      80      90      100     110     120
GACCGGATAG GCTCGCATCA CTACTTTTCAT GGTATCAAGC AAGCACTCAA TGAGTGCGGT

      130     140     150     160     170     180
GCCAGCOTCT TCOTTCCAAT CATTTTCAGCC GCCAACGACA ATGAAGCCCC AGGCGATCAA

10     190     200     210     220     230     240
CTGCTCAAGC AGATCCACAA CCTGCGCAGG CAGGTGCGGT CACAGCGTGT CAACCTGATC

      250     260     270     280     290     300
GGCCACAAGC AAGGCGCCCT TACCGCGCGT TATGTGGCGG CCATCGCCCC TGAAGTGATC

15     310     320     330     340     350     360
GCCTCGGTGA COTCAGTCAQ TGCCCCCAAC CACGGCTCCG AGCTGGCCGA TCGCTTGCGC

      370     380     390     400     410     420
CTGGCCTTTG TCCCGGGGAG GCTTGCCGAA ACGGTGCGTG CCGCCCTGAC CACCTCCTTC

      430     440     450     460     470     480
AGCGCATTTT TATCCGCCCT CAGCGGCCAC CCGGCGCTGC CCCAAAATGC CTTGAATGCG

25     490     500     510     520     530     540
CTTAACGCCC TGACCACCGA TGGGTTTCCG GCCTTCAACC GCCAATATCC CCAGGGGCTG

      550     560     570     580     590     600
CCCGACAAGT GGGGCGGCAT GGGCCCCGCA CAGGTGAATG CTGTGCACTA TTATTCCTGG

30     610     620     630     640     650     660
AGCGGCATCA TCAAAGGCTC GCGGCTGGCC GAGTCACTCA ACCTGCTCGA CCCCTTGAC

      670     680     690     700     710     720
AATGCCTTGC GTGTGTTTGA CAGCTTTTTT ACCCGCGAAA CCCGAGAAAA CGACGGCATG

35     730     740     750     760     770     780
GTTGGCCGCT TCAGCTCGCA TCTGGGCCAG GTCATCCGCT CCGACTACCC TCTGGACCAC

      790     800     810     820     830     840
CTCGACACCA TCAACCATAT GGCAAGAGGA AGCGCAGGCG CATCAACCCG GTAG

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2. A DNA arrangement according to claim 1 wherein said *Pseudomonas* genus bacterium is *Pseudomonas fragi* IFO 12049 strain.

3. A recombinant DNA replicable inside *Escherichia coli*, having a DNA arrangement coding said lipase originated from *Pseudomonas* genus bacterium or a DNA arrangement coding a functional equivalent to said nucleotide arrangement, each confirmed in claim 1, incorporated into a vector for *Escherichia coli*

4. A recombinant DNA according to claim 3, wherein said *Pseudomonas* genus bacterium is *Pseudomonas fragi* IFO 12049 strain.

5. A recombinant DNA according to claim 3, wherein said vector for *Escherichia coli* is pUC9.

6. A recombinant DNA according to claim 3, wherein said *Pseudomonas* genus bacterium is *Pseudomonas fragi* IFO 12049 strain and said vector for *Escherichia coli* is pUC9.

7. An *Escherichia coli* transformed by a recombinant DNA according to claim 3.

8. An *Escherichia coli* transformed by a recombinant DNA according to claim 3 wherein said *Pseudomonas* genus bacterium is *Pseudomonas fragi* IFO 12049.

9. An *Escherichia coli* transformed by a recombinant DNA according to claim 3 wherein said vector for *Escherichia coli* is pUC9.

10. A process for producing a lipase which comprises using a recombinant DNA according to claim 3.

11. A process for producing a lipase which comprises cultivating an *Escherichia coli* according to claim

5 7.

12. A process for producing a lipase which comprises cultivating an *Escherichia coli* according to claim 7 wherein said *Pseudomonas* genus bacterium is *Pseudomonas fragi* IFO 12049.

13. A process for producing a lipase which comprises cultivating an *Escherichia coli* according to claim 7 wherein said vector for *Escherichia coli* is pUC9.

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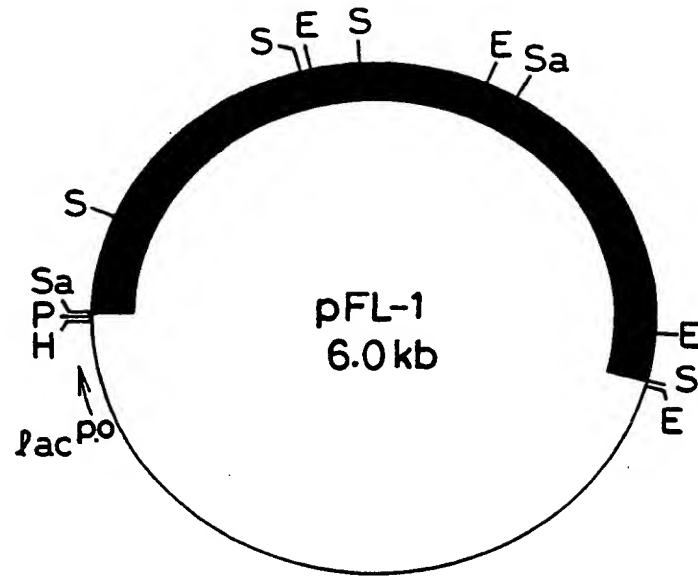
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## FIG.1

10	20	30	40	50	60
ATGGACGATT	CGGTAAATAC	ACGCTATCCG	ATCTTATTGG	TACACGGCCT	TTTTGGATTG
70	80	90	100	110	120
GACCGGATAG	GCTCGCATCA	CTACTTTTCAT	GGTATCAAGC	AAGCACTCAA	TGAGTGCGGT
130	140	150	160	170	180
GCCAGCGTCT	TCGTTCCAAT	CATTTTCAGCC	GCCAACGACA	ATGAAGCCCG	AGGCGATCAA
190	200	210	220	230	240
CTGCTCAAGC	AGATCCACAA	CCTGCGCAGG	CAGGTCGGTG	CACAGCGTGT	CAACCTGATC
250	260	270	280	290	300
GGCCACAGCC	AGGGCGCCCT	TACCGCGCGT	TATGTGGCGG	CCATCGCCCC	TGAACTGATC
310	320	330	340	350	360
GCCTCGGTGA	CGTCAGTCAG	TGGCCCCAAC	CACGGCTCCG	AGCTGGCCGA	TCGCTTGCGC
370	380	390	400	410	420
CTGGCCTTTG	TCCCGGGGAG	GCTTGCGCAA	ACGGTGGCTG	CCGCCCTGAC	CACCTCGTTC
430	440	450	460	470	480
AGCGCATTTT	TATCCGCCCT	CAGCGGCCAC	CCGCGCCTGC	CCCAAAATGC	CTTGAATGCG
490	500	510	520	530	540
CTTAACGCCC	TGACCACCGA	TGGGGTTGCC	GCCTTCAACC	GCCAATATCC	CCAGGGGCTG
550	560	570	580	590	600
CCCGACAGAT	GGGGCGGCAT	GGGCCCCGCA	CAGGTGAATG	CTGTGCACTA	TTATTCCTGG
610	620	630	640	650	660
AGCGGCATCA	TCAAAGGCTC	GCGGCTGGCC	GAGTCACTCA	ACCTGCTCGA	CCCCTTGAC
670	680	690	700	710	720
AATGCCTTGC	GTGTGTTTGA	CAGCTTTTTT	ACCCGCGAAA	CCCGAGAAAA	CGACGGCATG
730	740	750	760	770	780
GTTGGCCGCT	TCAGCTCGCA	TCTGGGCCAG	GTCATCCGCT	CCGACTACCC	TCTGGACCAC
790	800	810	820	830	840
CTCGACACCA	TCAACCATAT	GGCAAGAGGA	AGCGCAGGCG	CATCAACCCG	G TAG

FIG. 2



E: EcoRI , H: Hind III , S: SmaI , Sa: SalI

FIG. 3

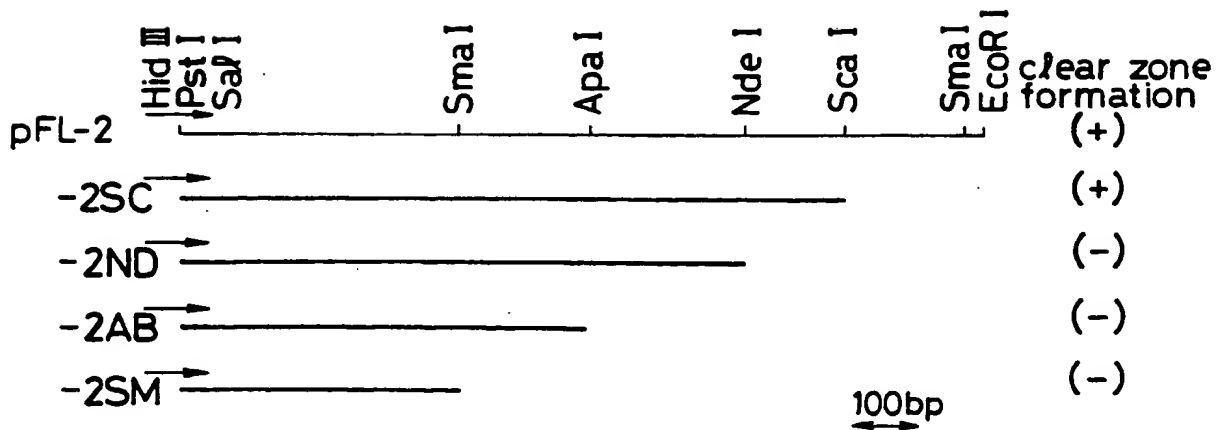
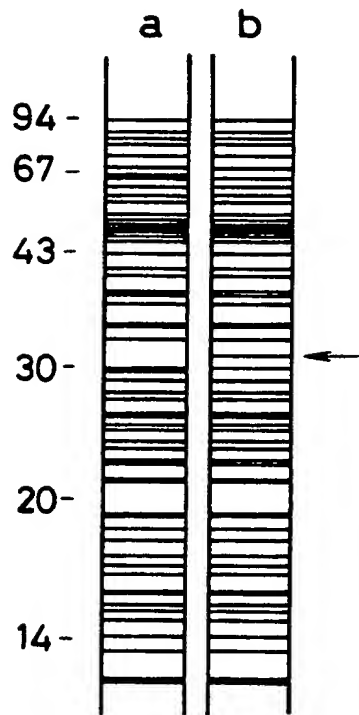


FIG. 4

10  
 ATGACGATTCGGTAATACACCGCTATCCGATCTTATTGGTACACGGCCCTTTTGGATTCCACCGGATAGGCTCGCATCACTTCTTATGGTATCAAGCAAGCACTCAATGAGTGGGT  
 M D D S V N T R Y P I L L V H G L F G F D R I G S H Y F H G I K Q A L N E C G  
 20  
 30  
 40  
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 60  
 70  
 80  
 90  
 100  
 110  
 120  
 AAGCTTGGCTGCAGGTCGACGGATCAAAACACCCCTCGGAGATTGAAC  
 130  
 140  
 150  
 160  
 170  
 180  
 190  
 200  
 210  
 220  
 230  
 240  
 GCCAGCGTCTTCGTTCCCATCTTTCAGCGCGCCCAACGACATGAAGCCCGGAGGCGATCACTGCTCAAGCAGATCCACAACTGCGCAGGCGAGGTGCGTGCACAGCGGTGTCAACCTGATC  
 A S V F V P I I S A A N D N E A R G D Q L L K Q I H N L R R Q V G A Q R V N L I  
 250  
 260  
 270  
 280  
 290  
 300  
 310  
 320  
 330  
 340  
 350  
 360  
 GGCACAGCCAGGCGCGCTTACCGCGCGTTATGTGGCGGCGATCGCGCCCTGAACCTGATCGCTCGGTGAGTCAAGTGGCGCCCAACCAAGCGGTCCAGGTGGCGCGATCGCTTGGCG  
 G H S Q G A L T A R Y V A A I A P E L I A S V T S V S G P N H G S E L A D R L R  
 370  
 380  
 390  
 400  
 410  
 420  
 430  
 440  
 450  
 460  
 470  
 480  
 CTGGCCCTTGTCCGGGGAGGGTTGGCGAAACGGGTGGCTGGCGCCCTGACCACTCTGTTCAGCGGATTTTATCCGCCCTCAGCGGCACCCCGCGCTGCCCAAAATGCCCTTGAATGCG  
 L A F V P G R L G E T V A A A L T T S F S A F L S A L S G H P R L P Q N A L N A  
 490  
 500  
 510  
 520  
 530  
 540  
 550  
 560  
 570  
 580  
 590  
 600  
 CTTAAGCCCTGACACCGCATGGGTTGCCCGCTTCAACCGCGCAATATCCCCAGGGGCTCCCGACACAGATGGGGCGGCATGGCGCCGACAGGTGAATGCTGCACTATTATTCCTGG  
 L N A L T T D G V A A F N R Q Y P Q G L P D R W G G M G P A Q V N A V H Y Y S W  
 610  
 620  
 630  
 640  
 650  
 660  
 670  
 680  
 690  
 700  
 710  
 720  
 AGCGGCATCATCAAGGCTCGCGGCTGGCGGAGTCACTCAACCTGCTCAGCCCTTGACAAATGCCCTTGCCTGTGTTTGACAGCTTTTATCCCGCAACCGGAGAAACGACGCGCATG  
 S G I I K G S R L A E S L N L L D P L H N A L R V F D S F F T R E T R E N D G M  
 730  
 740  
 750  
 760  
 770  
 780  
 790  
 800  
 810  
 820  
 830  
 GTTGGCCGCTTACGCTCGCATCTGGGCGAGGTGATCGGCTCGGACTAGCCTCTGGACCACTCAGCACCATCAAGCATATGGCAAGAGGAGGCGGCGCATCAACCCGGGTAGAGCTTT  
 V G R F S S H L G Q V I R S D Y P L D H L D T I N H M A R G S A G A S T R +  
 AATCGAGGACGGCAAGCGGCTGAAGGAGCGGCTGTAGGCTGTAAATCAATTCAAGCGGCGATTTGCCGAGAAATAATCACCAGATCCGGTAGGCTCAGCACCTTATTCAAGGA  
 GTATCTCCTCATGCGCAAGGCCGCTGCCGCTCAGATGCTGGTTGGCACCGGACAGCAAGTGCACGAACTGAAGCGGCAATCGAAGCGGCGCTGACTTCGCCCGAAGTTGCCAAAGCCCAAT  
 TCTCTTGGCCGCTCAGGCGGCGGACCTGGGCTCTTTGGGCGCGGCGCAGATGGTCAAGGAATT

FIG. 5



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European Patent Office  
Office européen des brevets

11 Publication number:

**0 318 775**  
**A3**

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## EUROPEAN PATENT APPLICATION

21 Application number: **88119211.6**

51 Int. Cl.<sup>5</sup>: **C12N 15/00, C07H 21/04,**  
**C12N 1/20, C12N 9/18,**  
**/(C12N1/20,C12R1:19)**

22 Date of filing: **18.11.88**

30 Priority: **03.12.87 JP 306638/87**  
**20.05.88 JP 123672/88**  
**27.07.88 JP 187684/88**

43 Date of publication of application:  
**07.06.89 Bulletin 89/23**

84 Designated Contracting States:  
**BE CH DE FR GB LI**

88 Date of deferred publication of the search report:  
**30.05.90 Bulletin 90/22**

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54 **A lipase gene.**

57 A process for producing a lipase is provided, which process comprises cloning the structural gene of a lipase present in the chromosome of Pseudomonas genus bacterium, determining the DNA sequence coding for the lipase, obtaining a recombinant DNA containing the DNA sequence and using Escherichia coli containing the recombinant DNA to obtain a lipase originating from Pseudomonas genus bacterium or its variant, suitable for commercial use, the DNA coding for the lipase having the following sequence:

**EP 0 318 775 A3**



DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
X,D	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 141, no. 1, 26th November 1986, pages 185-190, Academic Press, Inc., Duluth, MN, US; W. KUGIMIYA et al.: "Molecular cloning and nucleotide sequence of the lipase gene from Pseudomonas fragi" * Whole document *	1-14	C 12 N 15/00 C 07 H 21/04 C 12 N 1/20 C 12 N 9/18 // (C 12 N 1/20 C 12 R 1:19 )
A	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 51, no. 1, January 1987, pages 181-186, Tokyo, JP; T. NISHIO et al.: "Purification and some properties of lipase produced by Pseudomonas fragi 22.39 B"		
T	FEBS LETTERS, vol. 242, no. 1, 19th December 1988, pages 36-40, Elsevier, Amsterdam, NL; S. AOYAMA et al.: "Cloning, sequencing and expression of the lipase gene from Pseudomonas fragi IFO-12049 in E. coli" * Discussion *		
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
			C 12 N C 12 P
Place of search		Date of completion of the search	Examiner
THE HAGUE		23-01-1990	VAN PUTTEN A.J.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		I : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	